**REPLY UNDER 37 CFR 1.116-EXPEDITED PROCEDURE-
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REMARKS

Claims 16, 20-22, 24-31, 33-35, and 38-40 are currently pending. Claims ~~16 and~~ 34 have been amended to recite a method of administration by parenteral injection and to more precisely claim the derivatives of the peptide stabilizers. Support for this amendment can be found, e.g., in paragraphs 27, 28 and 38 of the patent application as filed. No new matter has been added. In the Final Office action, the Office indicated that claims 38-40 were withdrawn from further consideration as being drawn to a nonelected species.

Applicants wish to thank Examiner Russel for the courtesy of a telephonic interview on April 6, 2006. The substance of the interview was as described in the Examiner's interview summary.

Regarding clarification of the meaning of the term "alpha-keto" as addressed in the advisory action, enclosed is Chapter 21: Amino acid Degradation and Urea Cycle from Biochemistry, 3rd ed., Stryer, Stanford University, W.H. Freeman & Co., New York; (1988). As explained therein, alpha-keto derivatives of amino acids are naturally occurring compounds obtained by transamination and oxidative deamination of amino acids, the 2 major pathways for amino acid degradation by removal of the alpha-amino nitrogen. Alpha keto derivatives of amino acids bear a keto function on the alpha carbon of the amino acid, as shown for example at the bottom of page 495. **Therefore, alpha-keto forms of the claimed peptide stabilizers have an alpha-amino substituent which has been replaced by a keto substituent on the alpha carbon of an N-terminal amino acid in the dipeptide or tripeptide.**

Applicants confirm that the Information Disclosure Statement dated March 29, 2005 included only one sheet of references.

1. **Rejection of the Claims under 35 U.S.C. §103(a) (¶3)**

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PATENT

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Reconsideration is respectfully requested of the rejection of claims 16, 21, 22, 24-26, 30, 31, 33, 34 and 37 under 35 U.S.C. § 103(a) as being obvious over Sato, et al. (U.S. Patent Application Publication No. 2003/0092622).

Claim 16 is directed to a method of administering a stable pharmaceutical composition by parenteral injection, wherein the composition comprises erythropoietin and a peptide stabilizer, which is free of serum albumin. The peptide stabilizer is selected from the group consisting of Gly-Gly, Gly-Gly-Gly, Gly-Tyr, Gly-Phe, Gly-His, Gly-Asp, Gly-Ala, Ala-Gly, Ala-Ala, derivatives thereof and mixtures thereof. **As currently amended, the derivatives are selected from acylated, fluorinated, alpha-keto and salt forms of Gly-Gly, Gly-Gly-Gly, Gly-Tyr, Gly-Phe, Gly-His, Gly-Asp, Gly-Ala, Ala-Gly, Ala-Ala, or nitro Phe or p-amino Phe in place of Phe within Gly-Phe, or cyclohexyl Ala in place of Ala within Gly-Ala, Ala-Gly, or Ala-Ala.**

Sato et al. describe a protein formulation containing a stabilizer selected from tryptophan, a tryptophan derivative or a salt thereof. The addition of the stabilizer is said to promote long-term storage stability of the protein formulation. One of the proteins that may be stabilized using the method described by Sato et al. is erythropoietin.

As discussed above, the only stabilizers that Sato et al. describe are tryptophan, tryptophan derivatives, and salts thereof. Paragraph [0047] of Sato, et al. lists a large number of salts and derivatives that can be used which always comprise the amino acid tryptophan or a derivative or salt thereof. Sato et al. do not suggest the use of any of the peptides listed in claim 16. In addition, there is no suggestion or motivation in Sato, et al. to use any of the peptides listed in claim 16 as stabilizers.

The Office indicated that the obviousness rejection over Sato et al. was maintained in view of the fact that the Trp-based stabilizers of US 2003/0092622 and WO 01/64241 can be considered to be derivatives of the peptide stabilizers described in the present invention. Specifically, the Office has stated that the tryptophan-based stabilizers of Sato et al. constitute "derivatives" of the peptide stabilizers claimed in the present application because of similarity in structure (i.e., dipeptides or tripeptides having

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at least one amino acid in common) and ability to stabilize protein compositions. In support of its argument, the Office cited three patents and patent publications: U.S. Patent No. 6,624,289 (Bajaj), U.S. Patent No. 6,538,028 (Pierson et al.) and U.S. Patent Application No. 2005/0288222 (Heyward et al.). According to the Office, these documents were used to show that the term "derivative" is interpreted broadly in the art and that a peptide derivative does not have to have the same number of amino acids as the peptide or even be a peptide, i.e., it could be a non-peptidic derivative.

Applicants have amended independent claims 16 and 34 to further define derivatives as those which are acylated, fluorinated, alpha-keto or salt forms of the claimed peptide stabilizers, or as nitro Phe, p-amino Phe or cyclohexyl Ala. The derivatives now claimed do not include, for example, the Trp-Gly tryptophan derivative of Sato et al.

The specification of the present application states the following about the derivatives that can be used:

> Non-naturally occurring amino acids include, but are not limited to, amino acid derivatives and analogs. **Non-limiting examples of amino acid derivatives include** selenomethionine, telluro-methionine, and p-aminophenylalanine, **fluorinated amino acids** (e.g., fluorinated tryptophan, tyrosine and phenylalanine), **nitrophenylalanine**, nitrobenzoxadiazolyl-L-lysine, deoxymethylarginine, and **cyclohexylalanine**. Amino acid analogs include chemically synthesized compounds having properties known in the art to be characteristic of amino acids, examples of which include, e.g., the tryptophan "analog" b-selenolo[3,2-b]pyrrolylalanine and the proline "analog" thiaproline (1,3-thiazolidine4-carboxylic acid). **Additional amino acid derivatives include amino acid salts, acylated amino acids, and alpha-keto amino acids.**
>
> By way of example and not of limitation, a dipeptide can contain an L-amino acid and a D-amino acid, an L-amino acid and an amino acid salt, a D-amino acid and an amino acid analog, an L-amino acid and an acylated amino acid, an L-amino acid and an alpha-keto amino acid, an acylated amino acid and an alpha-keto amino acid, two acylated amino acids, two L-amino acids, two amino acid salts, etc. (Paragraphs 27 & 28 of the patent application publication; emphasis added)

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Therefore, one skilled in the art would recognize that the derivatives of the pending non-withdrawn claims are dipeptides or tripeptides which contain one or more amino acids salts, acylated amino acids, and alpha-keto amino acids. By way of example and as indicated above, derivatives of dipeptides such as, e.g., Gly-Gly, Gly-Tyr, Gly-Phe can include, e.g., glycine and glycine salt, glycine and fluorinated tyrosine, and glycine and p-aminophenylalanine, respectively. As another example, the derivatives of these peptides can include glycine salt and acylated glycine, alpha-keto glycine and tyrosine, and glycine and phenylalanine salt, respectively.

Based on the foregoing, applicants submit that the derivatives of the peptide stabilizers of the present invention are different than the peptide stabilizers of Sato et al. Furthermore, applicants submit that based on the teachings of Sato et al., a skilled artisan would not have been motivated to use the peptides or peptide derivatives of claim 16 to stabilize erythropoietin. In addition, there would have been no reasonable expectation that the peptides or peptide derivatives of claim 16 could be used successfully as erythropoietin stabilizers based on Sato et al. teachings.

With respect to serum albumin, Sato et al. state that the formulations may be substantially free of serum albumin, whereas the formulation of claim 16 is free of serum albumin. The Office noted that it would have been *prima facie* obvious to omit serum albumin from Sato et al. formulations because it is preferable in pharmaceutical arts to minimize the number of ingredients in order to reduce chances for any side effects. However, serum albumin has been used in protein formulations, including erythropoietin, because it stabilized the protein against physical and chemical changes that the protein can undergo in solution. Thus, even if one skilled in the art were motivated to remove serum albumin to minimize the number of components in a pharmaceutical formulation, that skilled person would not have a reasonable expectation of success in maintaining the stability of the formulation if the stabilizer is entirely removed. Accordingly, for all of the above reasons, applicants submit that claim 16 is non-obvious and patentable over Sato et al.

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Claims 21, 22, 24-26, 30, 31 and 33 depend either directly or indirectly from claim 16 and are thus patentable for the same reasons as claim 16, as well as for the additional elements they require.

Claim 34 is similar to amended claim 16, and further comprises a polyoxyalkylene sorbitan fatty acid ester. Claim 34 is patentable for the same reasons as set forth above for claim 16, as well as for the additional elements it requires.

2. **Rejection of the Claims Under 35 U.S.C. §103(a) (¶4)**

Reconsideration is respectfully requested of the rejection of claims 24-29 and 35 under 35 U.S.C. § 103(a) as being obvious over Sato, et al. (U.S. Patent Application Publication No. 2003/0092622) in view of WO 02/14356.

Claims 24-29 and 35 depend either directly or indirectly from claims 16 and 34, respectively, as discussed above.

Sato et al. is discussed above. WO 02/14356 discloses the preparation of erythropoietin omega and methods of treatment using the same.

The Office alleged that it would have been obvious to stabilize the erythropoietin omega of WO 02/14356 with a peptide stabilizer described by Sato, et al. to preserve its therapeutic activities.

WO 02/14356 does not teach or even mention that erythropoietin omega can be formulated using peptide stabilizers. There is therefore no motivation in the cited references to combine the teachings of Sato, et al. and WO 02/14356, absent the hindsight analysis of the applicants' disclosure. The Office alleged that the motivation to combine references can be found in the prior art as a whole because WO 02/14356 teaches a specific form of erythropoietin and Sato et al. list that erythropoietin can be stabilized with tryptophan, derivative thereof or salt thereof. Applicants note that even if the teachings of Sato et al. were combined with the teachings of WO 02/14356, one skilled in the art would still not have arrived at the present invention, i.e., a stable pharmaceutical composition of erythropoietin comprising a peptide stabilizer selected

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from the group consisting of Gly-Gly, Gly-Gly-Gly, Gly-Tyr, Gly-Phe, Gly-His, Gly-Asp, Gly-Ala, Ala-Gly, Ala-Ala, derivatives thereof and mixtures thereof, which is free of serum albumin. Sato et al. and WO 02/14356 thus either alone or in combination fail to teach or suggest all the limitations of the claims. Claims 24-29 and 35 are thus patentable over the combination of Sato et al. and WO 02/14356.

3. **Rejection of the Claims Under 35 U.S.C. §103(a) (¶5)**

Reconsideration is respectfully requested of the rejection of claims 16, 21, 22, 24-26, 30, 31, 33, 34, and 37 under 35 U.S.C. §103(a) as being obvious over WO 01/64241. As stated in the Office action, WO 01/64241 is the equivalent to the Sato et al. reference discussed above, only published in Japanese. Applicants therefore submit that claims 16, 21-22, 24-26, and 30-34 are patentable over WO 01/64241, for the same reasons as set forth above in §1 of this response, with respect to Sato et al.

4. **Rejection of the Claims Under 35 U.S.C. §103(a) (¶6)**

Reconsideration is respectfully requested of the rejection of claims 24-29 and 35 under 35 U.S.C. §103(a) as being obvious over WO 01/64241 in view of WO 02/14356. As mentioned above, WO 01/64241 is equivalent to the Sato et al. reference discussed above, only published in Japanese. Applicants therefore submit that claims 24-29 and 35 are patentable over WO 01/64241 in view of WO 02/14356, for the same reasons as set forth above in §2 of this response, with respect to Sato et al. and WO 02/14356.

5. **Rejection of the Claims Under 35 U.S.C. §103(a) (¶7)**

Reconsideration is respectfully requested of the rejection of claims 16, 20-22, 24, and 37 under 35 U.S.C. § 103(a) as being obvious over Cormier, et al. (U.S. Patent Application Publication No. 2002/0058608).

Cormier et al. teach a buffered aqueous formulation **for transdermal electrotransport delivery**, which comprises a therapeutic agent buffered with a dipeptide buffer. The Office has stated that it would have been obvious to administer

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EPO using the Gly-His buffer of Cormier et al. because the reference discloses that EPO is a protein which can be usefully administered in their formulations.

There is no teaching or suggestion in Cormier et al. of using their formulations for administration by parenteral injection, nor is there any teaching or suggestion of how a formulation for parenteral injection would be prepared or stabilized. In fact, if anything, Cormier et al. teach away from administration by parenteral injection. For example, Cormier et al. specifically point out drawbacks of parenteral injection, stating:

> Polypeptide and protein molecules are highly susceptible to degradation by proteolytic enzymes in the gastrointestinal tract and are subjected to an extensive hepatic metabolism when taken orally. Thus, these substances usually require parenteral administration to achieve therapeutic levels in the patient's blood. The most conventional parenteral administration techniques are hypodermic injections and intravenous administration. Polypeptides and proteins are, however, inherently short acting in their biological activity, requiring **frequent injections**, often several times a day, to maintain the therapeutically effective levels needed. Patients frequently find this treatment regimen to be **inconvenient and painful**. Such therapy also includes **risk of, e.g., infection**. (p. 1, ¶0007)

One skilled in the art would not have been motivated by Cormier et al. to parenterally inject the formulations of Cormier et al. Nor would Cormier et al. have provided any guidance to one of ordinary skill as to how to stabilize a parenteral injectable pharmaceutical composition containing erythropoietin since Cormier et al. would only guide one in selecting an appropriate pH stable buffer for transdermal electrotransport of a drug. To

establish *prima facie* obviousness of a claimed invention, all the claim limitations must be taught or suggested by the prior art. *In re Royka*, 490 F.2d 981, 180 USPQ 580 (CCPA 1974). Claim 16 is thus patentable over the cited reference as it fails to teach or suggest parenteral injection of such a composition.

Claims 20-22, and 24 depend either directly or indirectly from claim 16 and are therefore patentable over Cormier, et al. for the same reasons as set forth above for claim 16, as well as for the additional elements they require.

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6. **Rejection of the Claims Under 35 U.S.C. § 103(a) (¶8)**

Reconsideration is respectfully requested of the rejection of claims 24-29 under 35 U.S.C. § 103(a) as being obvious over Cormier et al. (U.S. Patent Application Publication No. 2002/0058608) and further in view of WO 02/14356.

Claims 24-29 depend either directly or indirectly from claim 16, which is discussed above. Cormier et al. and WO 02/14356 are discussed above.

The Office has alleged that it would have been obvious to formulate the erythropoietin omega of WO 02/14356 in the compositions of Cormier et al. because it would be desirable to administer erythropoietin omega iontophoretically, and Cormier et al. teach administration of a wide range of proteins. Claim 16 is patentable over Cormier et al. for the reasons set forth above. The deficiencies of Cormier et al. are not overcome by WO 02/14356 since one skilled in the art would not have been motivated to parenterally inject the composition of Cormier et al. given the teaching away as described in section 5 above. Applicants thus submit that claim 16 is patentable over Cormier et al. and WO 02/14356 either alone or in combination. Since claims 24-29 depend either directly or indirectly from claim 16, they are patentable for the same reasons as set forth above for claim 16.

7. **Rejection of the Claims Under 35 U.S.C. § 103(a) (¶9)**

Reconsideration is respectfully requested of the rejection of claims 30-31 and 33-34 under 35 U.S.C. §103(a) as being obvious over Cormier et al. (U.S. Patent Application Publication No. 2002/0058608) and further in view of Holladay et al. (U.S. Patent No. 6,328,728).

Claims 30-31 and 33 depend either directly or indirectly from claim 16, which is discussed above, and further call for the composition to comprise a surfactant.

Cormier, et al. is discussed above.

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Holladay et al. teach a method of enhancing electrotransport delivery of an active agent, such as a protein in the presence of at least one electrotransport enhancer selected from nonionic surfactants, zwitterionic surfactants lacking a net charge, and mixtures thereof, such as polyoxyethylene (20) sorbitan monolaurate or polyoxyethylene (20) sorbitan monopalmitate.

The Office has stated that it would have been obvious to one of ordinary skill in the art to include a surfactant of Holladay et al. in the compositions of Cormier et al. to increase the flux or decrease biodegradation of proteins during electrotransport delivery.

Claims 16 and 34 are patentable over Cormier et al. for the reasons set forth above. The deficiencies of Cormier et al. are not cured by the teachings of Holladay et al., since Holladay et al. merely teach enhancing electrotransport delivery of an active agent, such as a protein, in the presence of at least one electrotransport enhancer, such as certain surfactants. Motivation for parenteral injection of the composition of Cormier et al. can thus not be found in Holladay et al.

Claims 30-31 and 33 depend either directly or indirectly from claim 16 and are thus patentable for the same reasons as set forth above for claim 16, as well as for the additional elements they require.

8. **Rejection of the Claims Under 35 U.S.C. § 103(a) (¶10)**

Reconsideration is respectfully requested of the rejection of claim 35 under 35 U.S.C. §103(a) as being obvious over Cormier et al. (U.S. Patent Application Publication No. 2002/0058608) in view of WO 02/14356 and further in view of Holladay et al. (U.S. Patent No. 6,328,728).

Claim 35 depends from claim 34, discussed above, and further states that the erythropoietin is erythropoietin omega. For the reasons previously noted, claim 34 is patentable over Cormier et al. and Holladay et al. either alone or in combination.

The Office has stated that it would have been obvious to one of ordinary skill to include the surfactant of Holladay et al. in the composition of Cormier et al. as modified

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to include the EPO omega of WO 02/14356 to increase the flux or decrease biodegradation of proteins during electrotransport delivery. The deficiencies of Cormier et al. and Holladay et al. are not overcome by WO 02/14356 since one skilled in the art would not have been motivated to parenterally inject the composition of Cormier et al. given the teaching away as described in section 5 above. Applicants thus submit that claim 35 is patentable over Cormier et al., Holladay et al., and WO 02/14356 either alone or in combination.

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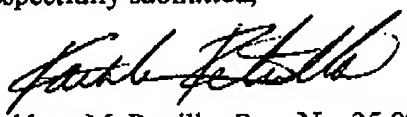
CONCLUSION

In view of the foregoing comments, Applicants respectfully request entry of the amendments and solicit allowance of the claims.

The Commissioner is hereby authorized to charge any deficiency or overpayment of the required fee to Deposit Account 19-1345.

Respectfully submitted,



Kathleen M. Petrillo, Reg. No. 35,076
SENNIGER POWERS
One Metropolitan Square, 16th Floor
St. Louis, Missouri 63102
(314) 231-5400

KMP/AXS/lam

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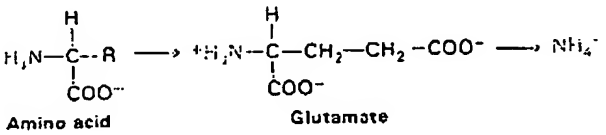
CHAPTER 21

Amino Acid Degradation and the Urea Cycle

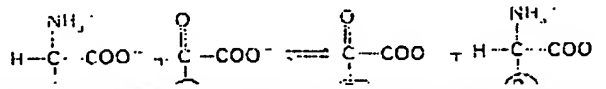
Amino acids in excess of those needed for the synthesis of proteins and other biomolecules cannot be stored, in contrast with fatty acids and glucose, nor are they excreted. Rather, surplus amino acids are used as metabolic fuel. *The α-amino group is removed and the resulting carbon skeleton is converted into a major metabolic intermediate.* Most of the amino groups of surplus amino acids are converted into urea, whereas their carbon skeletons are transformed into acetyl CoA, acetoacetyl CoA, pyruvate, or one of the intermediates of the citric acid cycle. Hence *fatty acids, ketone bodies, and glucose can be formed from amino acids.*

α-AMINO GROUPS ARE CONVERTED INTO AMMONIUM ION BY OXIDATIVE DEAMINATION OF GLUTAMATE

The major site of amino acid degradation in mammals is the liver. The fate of the α-amino group will be considered first, followed by that of the carbon skeleton. The α-amino group of many amino acids is transferred to α-ketoglutarate to form *glutamate*, which is then oxidatively deaminated to yield NH_4^+.



Aminotransferases catalyze the transfer of an α-amino group from an α-amino acid to an α-keto acid. These enzymes, also called *transami-*



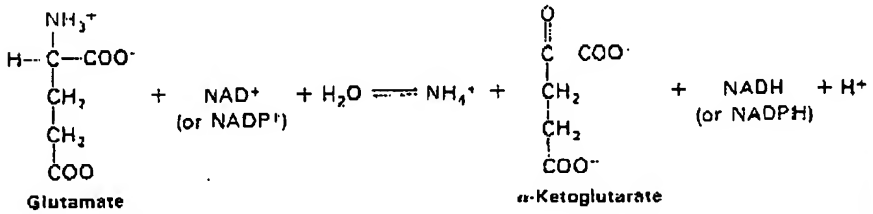
nases, generally *funnel α-amino groups from a variety of amino acids to α-ketoglutarate for conversion into* NH_4^+. *Aspartate aminotransferase*, one of the most important of these enzymes, catalyzes the transfer of the amino group of aspartate to α-ketoglutarate.

$$\text{Aspartate} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{oxaloacetate} + \text{glutamate}$$

Alanine aminotransferase, which is also prevalent in mammalian tissue, catalyzes the transfer of the amino group of alanine to α-ketoglutarate.

$$\text{Alanine} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{pyruvate} + \text{glutamate}$$

Ammonium ion is formed from glutamate by oxidative deamination. This reaction is catalyzed by *glutamate dehydrogenase*, which is unusual in being able to utilize either NAD^+ or $NADP^+$.

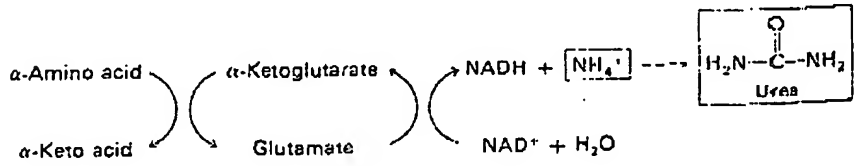


The activity of glutamate dehydrogenase is allosterically regulated. The vertebrate enzyme consists of six identical subunits, which can polymerize further. Guanosine triphosphate (GTP) and adenosine triphosphate (ATP) are allosteric inhibitors, whereas guanosine diphosphate (GDP) and adenosine diphosphate (ADP) are allosteric activators. Hence, *a lowering of the energy charge accelerates the oxidation of amino acids.*

The sum of the reactions catalyzed by aminotransferases and glutamate dehydrogenase is

$$\alpha\text{-Amino acid} + \underset{(\text{or } NADP^+)}{NAD^+} + H_2O \rightleftharpoons \alpha\text{-keto acid} + NH_4^+ + \underset{(\text{or NADPH})}{NADH} + H^+$$

In terrestrial vertebrates, NH_4^+ is converted into urea, which is excreted. The synthesis of urea will be discussed shortly.



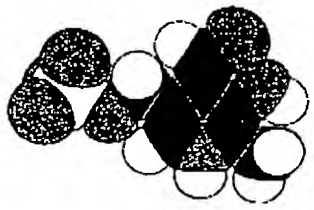
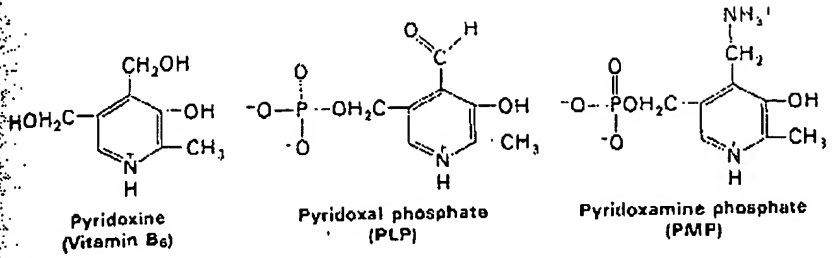


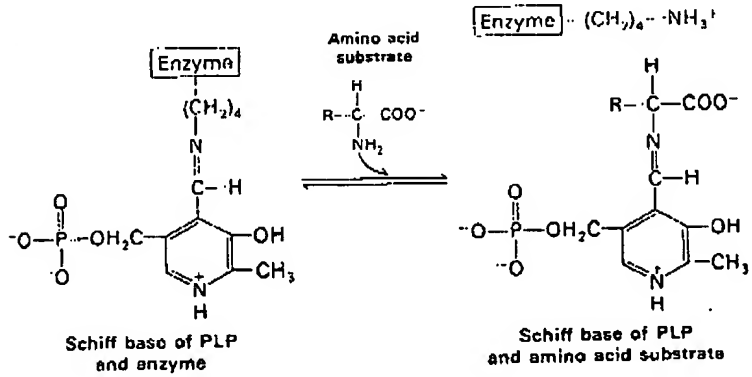
Figure 21-1
Space-filling model of pyridoxal 5-phosphate (PLP).

PYRIDOXAL PHOSPHATE FORMS SCHIFF-BASE INTERMEDIATES IN AMINOTRANSFERASES

The prosthetic group of all aminotransferases is *pyridoxal phosphate* (PLP), which is derived from *pyridoxine* (vitamin B_6). During transamination, pyridoxal phosphate is transiently converted into *pyridoxamine phosphate* (PMP).



PLP enzymes form covalent Schiff-base intermediates with their substrates. In the absence of substrate, the aldehyde group of PLP is in Schiff-base linkage with the *ε-amino group of a specific lysine residue at the active site*. A new Schiff-base linkage is formed on addition of an amino acid substrate. *The α-amino group of the amino acid substrate displaces the ε-amino group of the active-site lysine*. The amino acid–PLP Schiff base that is formed remains tightly bound to the enzyme by multiple noncovalent interactions.



Esmond Snell and Alexander Braunstein proposed more than forty years ago a reaction mechanism for transamination that has proven to be generally valid. The Schiff base between the amino acid substrate and PLP, termed an *aldimine*, loses a proton from its α-carbon to form a *quinonoid* intermediate. Reprotonation yields a *ketimine*, which contains a double bond between N and C_α of the substrate. In contrast, the aldimine contains a double bond between N and the carbonyl C of PLP.

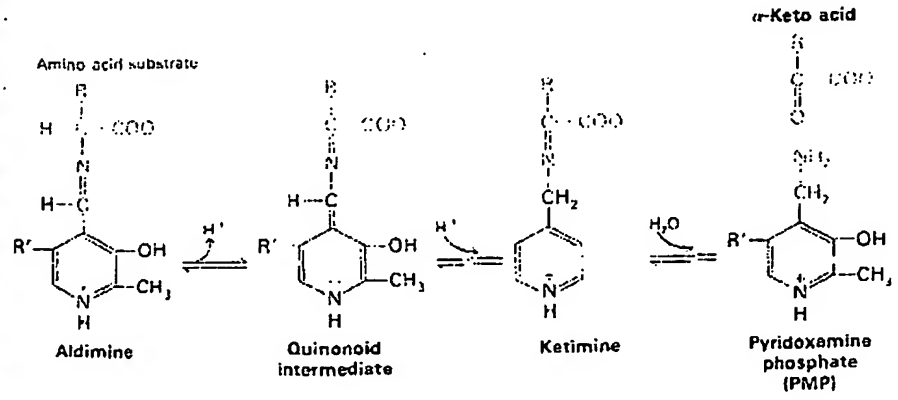


Figure 21-2
Proposed mechanism of transamination reactions.

The ketimine is then hydrolyzed to an α-keto acid and pyridoxamine phosphate. These steps comprise half of the transamination reaction.

$$\text{Amino acid}_1 + \text{E-PLP} \rightleftharpoons \alpha\text{-keto acid}_1 + \text{E-PMP}$$

The second half occurs by a reversal of the above pathway. A second α-keto acid reacts with the enzyme–pyridoxamine phosphate complex (E-PMP) to yield a second amino acid and regenerate the enzyme–pyridoxal phosphate complex (E-PLP).

$$\alpha\text{-Keto acid}_2 + \text{E-PMP} \rightleftharpoons \text{amino acid}_2 + \text{E-PLP}$$

The sum of these partial reactions is

$$\text{Amino acid}_1 + \alpha\text{-keto acid}_2 \rightleftharpoons \text{amino acid}_2 + \alpha\text{-keto acid}_1$$

THE ACTIVE SITE CLEFT OF ASPARTATE AMINOTRANSFERASE CLOSES WHEN SUBSTRATE FORMS A SCHIFF BASE LINKAGE

X-ray crystallographic studies of mitochondrial aspartate aminotransferase have provided detailed views of how PLP and substrate are bound and have confirmed much of the proposed catalytic mechanism. Each of the identical 45-kd subunits of this dimer consists of a large domain and a small one. PLP is bound to the large domain, in a pocket near the subunit interface (Figure 21-3). The pyridine nitrogen atom of

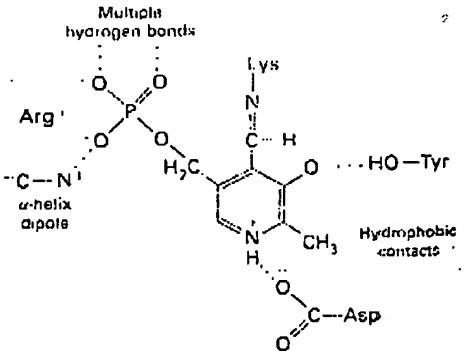


Figure 21-3
Mode of binding of PLP to aspartate aminotransferase. [After J. F. Kirsch, G. Eichele, G. F. Ford, M. G. Vincent, and J. N. Jansonius. *J. Mol. Biol.* 174(1984):510.]

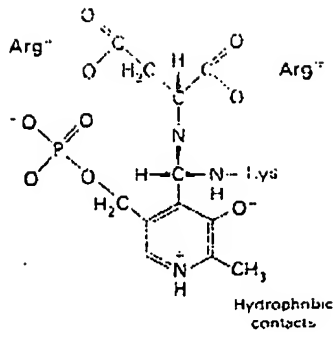


Figure 21-4
Mode of binding of aspartate in the tetrahedral intermediate of aspartate aminotransferase. [After J. F. Kirsch, G. Eichele, G. F. Ford, M. G. Vincent, and J. N. Jansonius. *J. Mol. Biol.* 174(1984):510.]

PLP is hydrogen-bonded to an aspartate carboxylate, and the 2-methyl group is in van der Waals contact with several hydrophobic residues. The 3-hydroxyl group is ionized and hydrogen bonded to the phenolic OH of a tyrosine. The 5-phosphate group of PLP is hydrogen bonded to seven groups. Its two negative charges are balanced by an arginine side chain and by the positive dipole at the amino end of an α helix. As was postulated earlier on the basis of spectroscopic and chemical studies, the 4-aldehyde group of PLP has been shown by x-ray crystallography to be in Schiff base linkage with a lysine residue.

Each of the two carboxylates of aspartate (or glutamate) forms a salt bridge with an arginine residue of the aminotransferase (Figure 21-4). These electrostatic interactions with guanido groups on different chains largely determine the substrate specificity of the enzyme. Moreover, they lead to a substantial movement of the small domain, which

closes the active-site crevice. Recall that hexokinase (p. 365) and citrate synthase (p. 384) also undergo cleft closure during catalysis. The subsequent replacement of the ε-amino group of lysine by the α-amino group of the substrate induces a 30-degree tilting of the PLP ring, which promotes the subsequent conversions. Specifically, the C_α–H bond becomes nearly perpendicular to the plane containing the PLP ring. This orientation facilitates the release of the α-H to form the quinonoid intermediate (see Figure 21-2). Reprotonation gives the ketimine, which is hydrolyzed to give oxaloacetate, the α-keto acid. The lysine amino group that was initially in Schiff base linkage with PLP serves as a proton acceptor and donor in subsequent steps.

PYRIDOXAL PHOSPHATE, A HIGHLY VERSATILE COENZYME, CATALYZES MANY REACTIONS OF AMINO ACIDS

Transamination is just one of a wide range of amino acid transformations that are catalyzed by PLP enzymes. The other reactions at the α-carbon atom of amino acids are decarboxylations, deaminations, racemizations, and aldol cleavages (Figure 21-5). In addition, PLP enzymes catalyze elimination and replacement reactions at the β-carbon atom (e.g., tryptophan synthetase, p. 586) and the γ-carbon atom (e.g., cystathionase, p. 584) of amino acid substrates. The common features of PLP catalysis underlying these diverse reactions are: (1) A *Schiff base* is formed by the amino acid substrate (the amine component) and PLP (the carbonyl component). (2) The protonated form of PLP acts as an *electron sink* to stabilize catalytic intermediates that are negatively charged—the ring nitrogen of PLP attracts electrons from the amino acid substrate. In other words, PLP is an *electrophilic catalyst.* (3) The product Schiff base is then hydrolyzed.

How does an enzyme selectively break one of three bonds at the α-carbon atom of an amino acid substrate? An important principle is that *the bond being broken must be perpendicular to the π-orbitals of the electron sink.* In an aminotransferase, for example, this is accomplished by binding the amino acid substrate so that the C_α–H bond is perpendicular to the PLP ring. This means of choosing one of several possible catalytic outcomes is called *stereoelectronic control.*

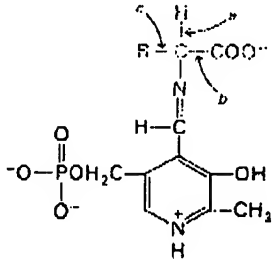


Figure 21-5
Pyridoxal phosphate enzymes labilize one of three bonds at the α-carbon atom of an amino acid substrate. For example, bond *a* is labilized by aminotransferases, bond *b* by decarboxylases, and bond *c* by aldolases (such as threonine aldolases). PLP enzymes also catalyze reactions at the β- and γ-carbon atoms of amino acids.

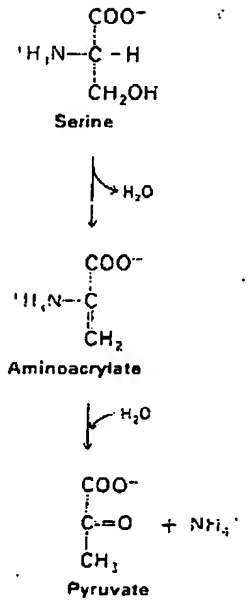
SERINE AND THREONINE CAN BE DIRECTLY DEAMINATED

The α-amino groups of serine and threonine can be directly converted into NH_4^+ because each of these amino acids contains a hydroxyl group attached to its β carbon atom. These direct deaminations are catalyzed by *serine dehydratase* and *threonine dehydratase,* in which PLP is the prosthetic group.

$$\text{Serine} \longrightarrow \text{pyruvate} + NH_4^+$$

$$\text{Threonine} \longrightarrow \alpha\text{-ketobutyrate} + NH_4^+$$

These enzymes are called dehydratases because dehydration precedes deamination. Serine loses a hydrogen atom from its α-carbon and a hydroxyl group from its β-carbon to yield aminoacrylate. This unstable compound reacts with H_2O to give pyruvate and NH_4^+.



NH_4^+ IS CONVERTED INTO UREA IN MOST TERRESTRIAL VERTEBRATES AND THEN EXCRETED

Some of the NH_4^+ formed in the breakdown of amino acids is consumed in the biosynthesis of nitrogen compounds. In most terrestrial vertebrates, the excess NH_4^+ is converted into urea and then excreted. In birds and terrestrial reptiles, NH_4^+ is converted into uric acid for excretion, whereas, in many aquatic animals, NH_4^+ itself is excreted. These three classes of organisms are called *ureotelic*, *uricotelic*, and *ammonotelic*.

In terrestrial vertebrates, urea is synthesized by the *urea cycle* (Figure 21-6). This series of reactions was proposed by Hans Krebs and Kurt Henseleit (a medical student) in 1932, five years before the elucidation of the citric acid cycle. In fact, the urea cycle was the first cyclic metabolic pathway to be discovered. One of the nitrogen atoms of the urea synthesized by this pathway is transferred from an amino acid, aspartate. The other nitrogen atom and the carbon atom are derived from NH_4^+ and CO_2. *Ornithine* is the carrier of these carbon and nitrogen atoms.

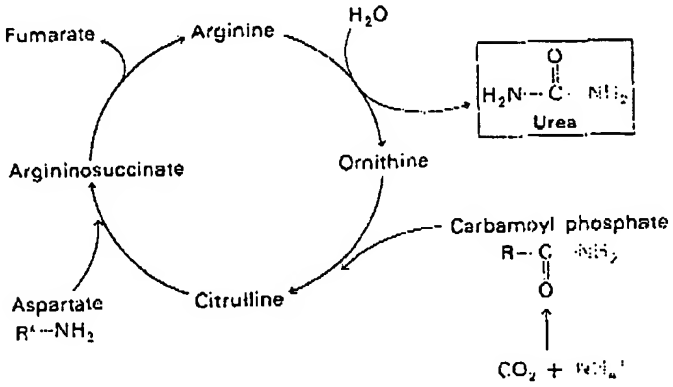
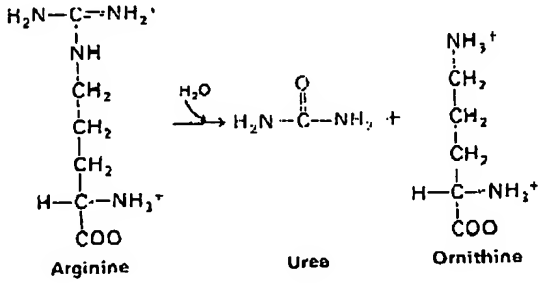
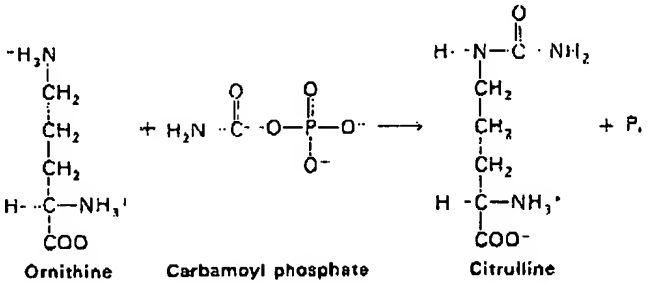


Figure 21-6
The urea cycle.

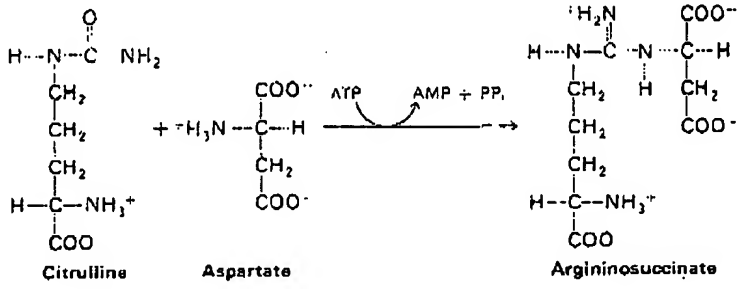
The immediate precursor of urea is *arginine*, which is hydrolyzed to urea and ornithine by *arginase*.



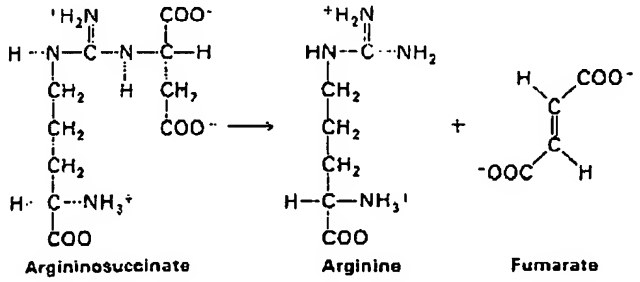
The other reactions of the urea cycle lead to the synthesis of arginine from ornithine. First, a carbamoyl group is transferred to ornithine to form *citrulline*, in a reaction catalyzed by ornithine transcarbamoylase. The carbamoyl donor in this reaction is carbamoyl phosphate, which has a high transfer potential because of its anhydride bond.



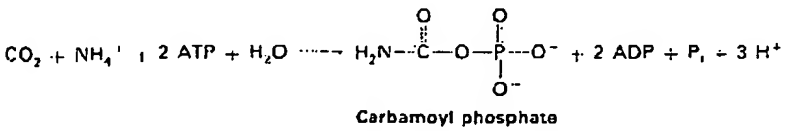
Argininosuccinate synthetase then catalyzes the condensation of citrulline and aspartate. This synthesis of *argininosuccinate* is driven by the cleavage of ATP into AMP and pyrophosphate and by the subsequent hydrolysis of pyrophosphate.



Finally, argininosuccinase cleaves argininosuccinate into *arginine* and *fumarate*. Note that these reactions, which transfer the amino group of aspartate to form arginine, preserve the carbon skeleton of aspartate.



Carbamoyl phosphate is synthesized from NH_4^+, CO_2, ATP, and H_2O in a complex reaction that is catalyzed by *carbamoyl phosphate synthetase*. An unusual feature of this enzyme is that it requires *N*-acetylglutamate for activity.



The consumption of two molecules of ATP makes this synthesis of carbamoyl phosphate essentially irreversible.

THE UREA CYCLE IS LINKED TO THE CITRIC ACID CYCLE

The stoichiometry of urea synthesis is

$$CO_2 + NH_4^+ + 3\ ATP + \text{aspartate} + 2\ H_2O \longrightarrow \text{urea} + 2\ ADP + 2\ P_i + AMP + PP_i + \text{fumarate}$$

Pyrophosphate is rapidly hydrolyzed, and so four ~P are consumed in these reactions to synthesize one molecule of urea. *The synthesis of fumarate by the urea cycle is important because it links the urea cycle and the citric acid cycle* (Figure 21-7). Fumarate is hydrated to malate, which is in turn oxidized to oxaloacetate. Oxaloacetate has several possible fates: (1) transamination to aspartate; (2) conversion into glucose by the gluconeogenic pathway; (3) condensation with acetyl CoA to form citrate; or (4) conversion into pyruvate.

The compartmentation of the urea cycle and its associated reactions is also noteworthy. The formation of NH_4^+ by glutamate dehydrogenase, its incorporation into carbamoyl phosphate, and the subsequent synthesis of citrulline occur in the mitochondrial matrix. In contrast, the next three reactions of the urea cycle, which lead to the formation of urea, take place in the cytosol.

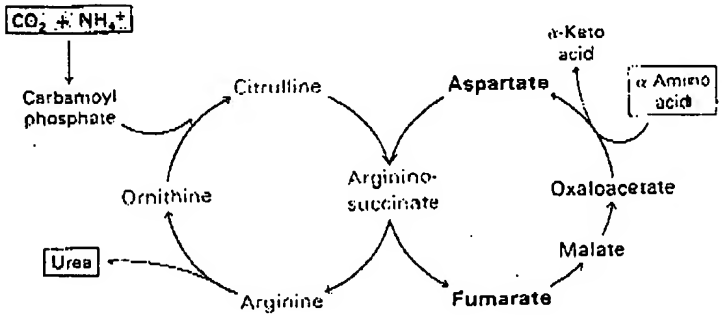
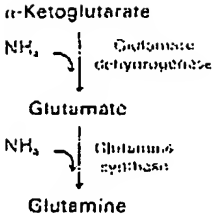


Figure 21-7
The urea cycle, the citric acid cycle, and the transamination of oxaloacetate are linked by fumarate and aspartate.

INHERITED ENZYMATIC DEFECTS OF THE UREA CYCLE CAUSE HYPERAMMONEMIA

High levels of NH_4^+ are toxic to humans. The synthesis of urea in the liver is the major route of removal of NH_4^+. A complete block of any of the steps of the urea cycle is probably fatal, because there is no known alternative pathway for the synthesis of urea. Inherited disorders caused by a partial block of each of the urea cycle reactions have been diagnosed. *The common condition is an elevated level of NH_4^+ in the blood (hyperammonemia).* A nearly total deficiency of any of the urea cycle enzymes results in coma and death shortly after birth. Partial deficiencies of these enzymes cause mental retardation, lethargy, and episodic vomiting. A low-protein diet leads to a lowering of the ammonium level in the blood and to clinical improvement in the milder forms of these inherited disorders.



Why are high levels of NH_4^+ toxic? A high concentration of ammonium ion shifts the equilibrium of the reaction catalyzed by glutamate dehydrogenase toward the formation of glutamate. NH_4^+ then reacts with glutamate to form glutamine (p. 577). Elevated levels of glutamine are found in the cerebrospinal fluid of patients with hyperammonemia and may lead directly to brain damage. This important question deserves further study.

CARBON ATOMS OF DEGRADED AMINO ACIDS EMERGE IN MAJOR METABOLIC INTERMEDIATES

Thus far, we have considered a series of reactions that removes the α-amino group from amino acids and converts it into urea. We now turn to the fates of the remaining carbon skeletons. *The strategy of amino acid degradation is to form major metabolic intermediates that can be converted into glucose or be oxidized by the citric acid cycle.* In fact, the carbon skeletons of the diverse set of twenty amino acids are funneled into only seven molecules: *pyruvate, acetyl CoA, acetoacetyl CoA, α-ketoglutarate, succinyl CoA, fumarate, and oxaloacetate.* We see here a striking example of the remarkable economy of metabolic conversions.

Amino acids that are degraded to acetyl CoA or acetoacetyl CoA are termed *ketogenic* because they give rise to ketone bodies. In contrast, amino acids that are degraded to pyruvate, α-ketoglutarate, succinyl CoA, fumarate, or oxaloacetate are termed *glucogenic.* Net synthesis of glucose from these amino acids is feasible because these citric acid cycle intermediates and pyruvate can be converted into phosphoenolpyruvate and then into glucose (p. 438). Recall that mammals lack a pathway for the net synthesis of glucose from acetyl CoA or acetoacetyl CoA.

Of the basic set of twenty amino acids, only leucine and lysine are purely ketogenic. Isoleucine, phenylalanine, tryptophan, and tyrosine are both ketogenic and glucogenic. Some of their carbon atoms emerge in acetyl CoA or acetoacetyl CoA, whereas others appear in potential precursors of glucose. The other fourteen amino acids are classed as purely glucogenic. This classification is not universally accepted because different quantitative criteria are applied. Whether an amino acid is regarded as being glucogenic, ketogenic, or both depends partly on the eye of the beholder.

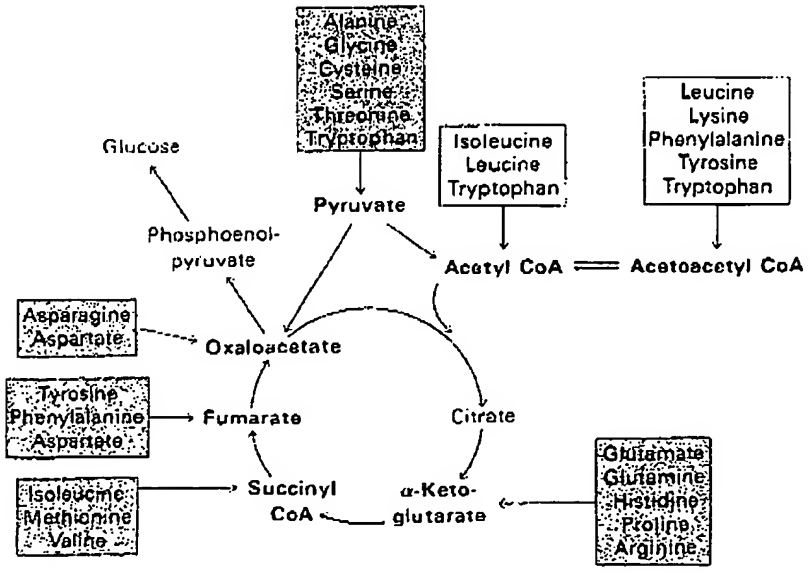


Figure 21-8
Fates of the carbon skeletons of amino acids. Glucogenic amino acids are shaded red, and ketogenic amino acids are shaded yellow.

THE C_3 FAMILY: ALANINE, SERINE, AND CYSTEINE ARE CONVERTED INTO PYRUVATE

Pyruvate is the entry point for the three-carbon amino acids: alanine, serine, and cysteine (Figure 21-9). The transamination of alanine directly yields pyruvate:

$$\text{Alanine} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{pyruvate} + \text{glutamate}$$

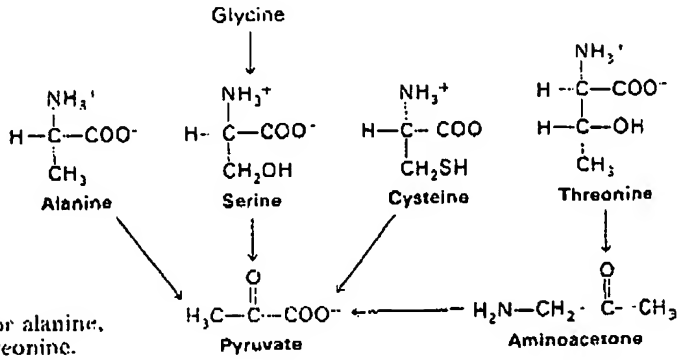


Figure 21-9
Pyruvate is the point of entry for alanine, serine, cysteine, glycine, and threonine.

As mentioned previously (p. 495), glutamate is then oxidatively deaminated, yielding NH_4^+ and regenerating α-ketoglutarate. The sum of these reactions is

$$\text{Alanine} + NAD^+ + H_2O \longrightarrow \text{pyruvate} + NH_4^+ + NADH + H^+$$

Another simple reaction in the degradation of amino acids is the *deamination of serine to pyruvate* by serine dehydratase (p. 499).

$$\text{Serine} \longrightarrow \text{pyruvate} + NH_4^+$$

Cysteine can be converted into pyruvate by several pathways, with its sulfur atom emerging in H_2S, SO_3^{2-}, or SCN^-.

The carbon atoms of three other amino acids can be converted into pyruvate. *Glycine* can be converted into serine by enzymatic addition of a hydroxymethyl group (p. 580). *Threonine* can give rise to pyruvate by way of aminoacetone. Three carbon atoms of *tryptophan* can emerge in alanine, which can be converted into pyruvate.

THE C_4 FAMILY: ASPARTATE AND ASPARAGINE ARE CONVERTED INTO OXALOACETATE

Aspartate, a four-carbon amino acid, is directly *transaminated to oxaloacetate*, a citric acid cycle intermediate:

$$\text{Aspartate} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{oxaloacetate} + \text{glutamate}$$

Asparagine is hydrolyzed by *asparaginase* to NH_4^+ and aspartate, which is then transaminated.

Recall that aspartate can also be converted into *fumarate* by the urea cycle (p. 500). Fumarate is also a point of entry for half of the carbon atoms of tyrosine and phenylalanine, as will be discussed shortly.

THE C_5 FAMILY: SEVERAL AMINO ACIDS ARE CONVERTED INTO α-KETOGLUTARATE THROUGH GLUTAMATE

The carbon skeletons of several five-carbon amino acids enter the citric acid cycle at *α-ketoglutarate*. These amino acids are first converted into *glutamate*, which is then oxidatively deaminated by glutamate dehydrogenase to yield α-ketoglutarate (Figure 21-10).

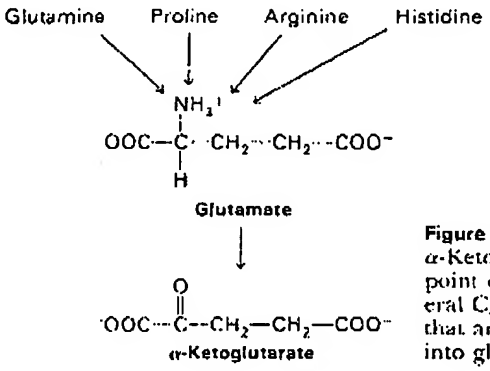


Figure 21-10
α-Ketoglutarate is the point of entry of several C_5 amino acids that are first converted into glutamate.

Histidine is converted into 4-imidazolone 5-propionate (Figure 21-11). The amide bond in the ring of this intermediate is hydrolyzed to the *N*-formimino derivative of glutamate, which is then converted into glutamate by transfer of its formimino group to tetrahydrofolate, a carrier of activated one-carbon units (see p. 580).

Glutamine is hydrolyzed to glutamate and NH_4^+ by glutaminase. *Proline* and *arginine* are converted into glutamate γ-semialdehyde, which is then oxidized to glutamate (Figure 21-12).

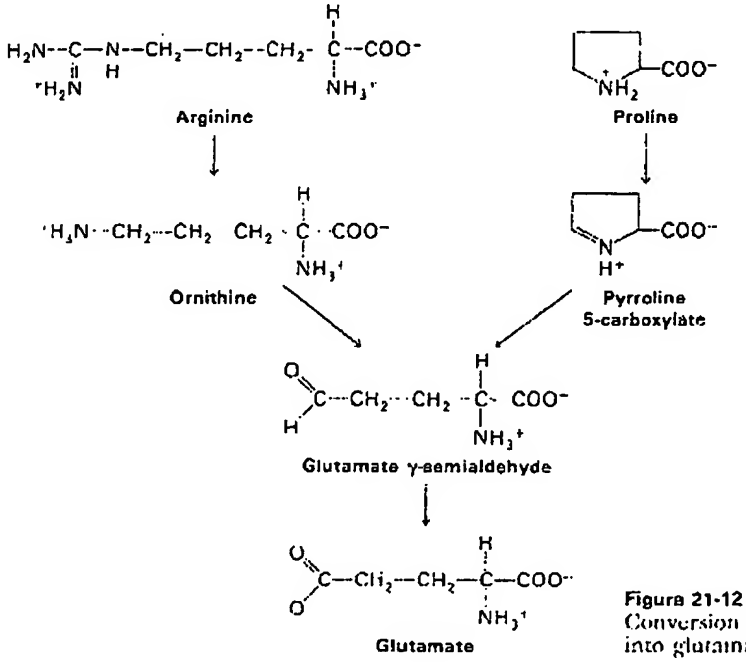


Figure 21-12
Conversion of proline and arginine into glutamate.

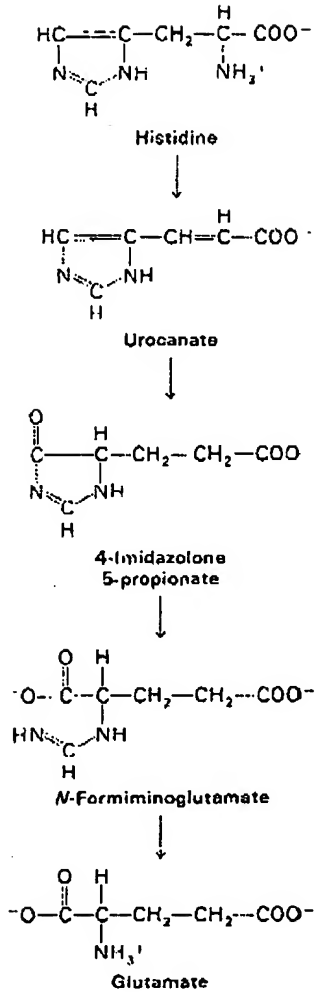


Figure 21-11
Conversion of histidine into glutamate.

SUCCINYL COENZYME A IS A POINT OF ENTRY FOR SOME AMINO ACIDS

Succinyl CoA is the point of entry for some of the carbon atoms of methionine, isoleucine, and valine. Methylmalonyl CoA is an intermediate in the breakdown of these three amino acids (Figure 21-13).

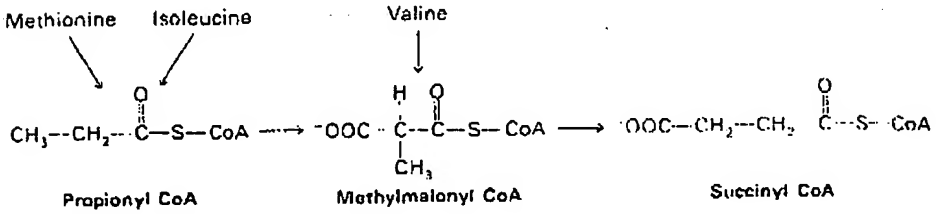
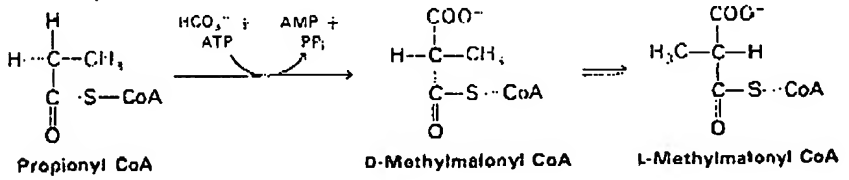
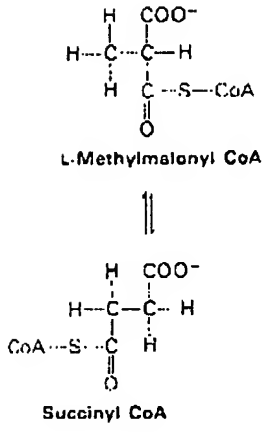


Figure 21-13
Conversion of methionine, isoleucine, and valine into succinyl CoA.

The pathway from propionyl CoA to succinyl CoA is especially interesting. Propionyl CoA is carboxylated at the expense of an ATP to yield the D-isomer of methylmalonyl CoA. This carboxylation reaction is catalyzed by *propionyl CoA carboxylase*, a biotin enzyme that has a catalytic mechanism like that of acetyl CoA carboxylase and pyruvate carboxylase. The D-isomer of methylmalonyl CoA is racemized to the L-isomer, which is the substrate for the mutase enzyme that converts it into succinyl CoA.





Succinyl CoA is formed from L-methylmalonyl CoA by an *intramolecular rearrangement*. The —CO—S—CoA group migrates from C-2 to C-3 in exchange for a hydrogen atom. *This very unusual isomerization is catalyzed by methylmalonyl CoA mutase, one of the two mammalian enzymes known to contain a derivative of vitamin B_{12} as its coenzyme.*

This pathway from propionyl CoA to succinyl CoA also participates in the oxidation of *fatty acids that have an odd number of carbon atoms*. The final thiolytic cleavage of an odd-numbered acyl CoA yields acetyl CoA and propionyl CoA (p. 478). Hence, odd-carbon fatty acids are partly glucogenic; specifically, three of their carbon atoms can emerge in glucose.

THE COBALT ATOM OF VITAMIN B_{12} IS BONDED TO THE 5'-CARBON OF DEOXYADENOSINE IN COENZYME B_{12}

Cobalamin (vitamin B_{12}) has been a challenging problem in biochemistry and medicine since the discovery by George Minot and William Murphy in 1926 that pernicious anemia can be treated by feeding the patient large amounts of liver. Cobalamin was first purified in 1948; and it was crystallized then by Dorothy Hodgkin, who elucidated its complex three-dimensional structure in 1956. The core of cobalamin consists of

a *corrin ring with a central cobalt atom* (Figure 21-14). The corrin ring, like a porphyrin, has *four pyrrole units*. Two of them (rings A and D) are directly bonded to each other, whereas the others are joined by methene bridges, as in porphyrins.

A cobalt atom is bonded to the four pyrrole nitrogens. *The fifth substituent* (below the corrin plane in Figure 21-15) is a derivative of *dimethylbenzimidazole* that contains ribose 3-phosphate and aminoisopropanol. One of the nitrogen atoms of dimethylbenzimidazole is linked to cobalt. The amino group of aminoisopropanol is in amide linkage with a side chain. The *sixth substituent* of the cobalt atom (located above the corrin plane in Figure 21-15) can be $-CH_3$, OH^-, or a 5′-deoxyadenosyl unit.

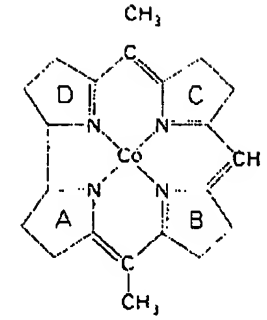


Figure 21-14
Corrin core of cobalamin (vitamin B_{12}). Substituents on the pyrroles and the other two ligands to cobalt are not shown in this diagram.

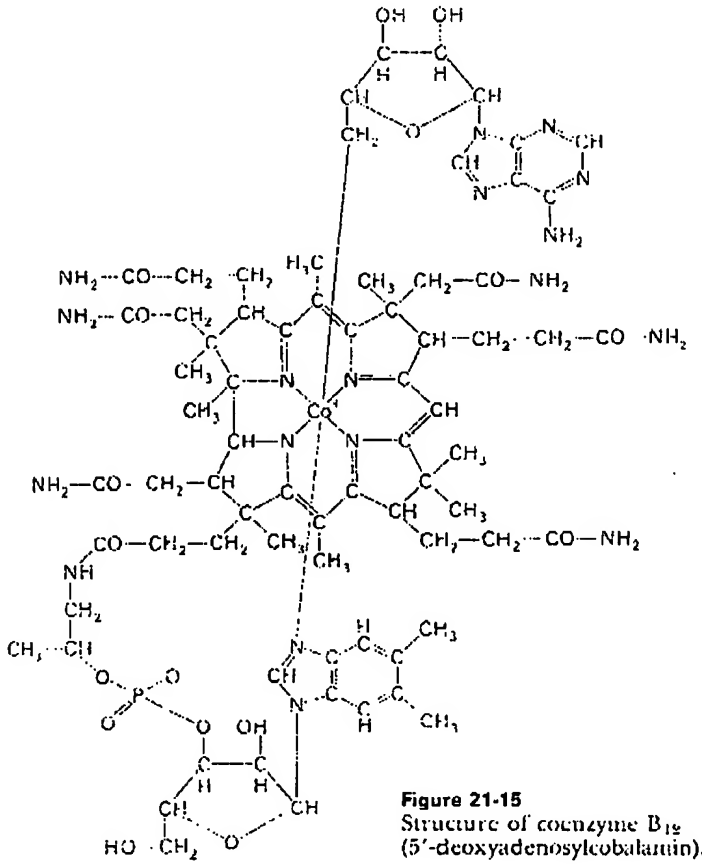


Figure 21-15
Structure of coenzyme B_{12} (5′-deoxyadenosylcobalamin).

The cobalt atom in cobalamin can have a +1, +2, or +3 oxidation state. The cobalt atom is in the +3 state in hydroxocobalamin (where OH^- occupies the sixth coordination site). This form, called B_{12a} (Co^{3+}), is reduced to a divalent state, called B_{12r} (Co^{2+}), by a flavoprotein reductase. The B_{12r} (Co^{2+}) form is reduced by a second flavoprotein reductase to B_{12s} (Co^{+}). NADH is the reductant in both reactions.

$$B_{12a}\ (Co^{3+}) \longrightarrow B_{12r}\ (Co^{2+}) \longrightarrow B_{12s}\ (Co^{+})$$

The B_{12s} form is the substrate for the final enzymatic reaction that yields the active coenzyme. Co^+ attacks the 5′-carbon atom of ATP and displaces the triphosphate group to form *5′-deoxyadenosylcobalamin*, also known as *coenzyme* B_{12} (Figure 21-16). This compound is remarkable in

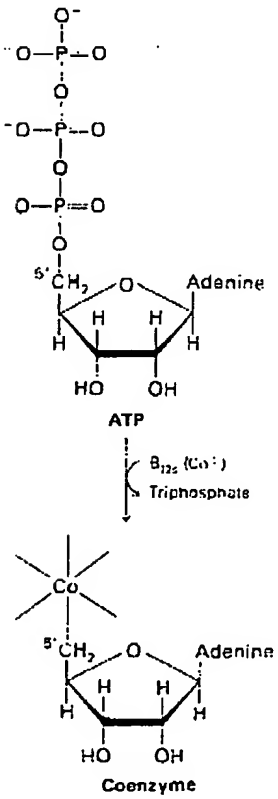


Figure 21-16
Formation of coenzyme B_{12} from cobalamin and ATP. The 5′ carbon of 5′-deoxyadenosine is coordinated to the cobalt atom in this coenzyme.

having a *carbon-metal bond*, the only one known in a biomolecule. Another unusual feature of this reaction is that the 5′-methylene carbon atom of ATP, rather than its α or β phosphorus atom, is the target of nucleophilic attack. The formation of *S*-adenosyl methionine (p. 582) is the only other biochemical reaction in which a nucleophile displaces the triphosphate group of ATP.

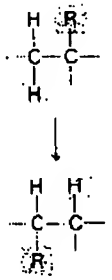


Figure 21-17
Rearrangement reaction catalyzed by cobalamin enzymes. The R group can be an amino group, a hydroxyl group, or a substituted carbon.

COENZYME B_{12} PROVIDES FREE RADICALS TO CATALYZE INTRAMOLECULAR MIGRATIONS INVOLVING HYDROGEN

Cobalamin enzymes catalyze three types of reactions: (1) *intramolecular rearrangements*; (2) *methylations*, as in the synthesis of methionine (p. 583); and (3) *reduction of ribonucleotides to deoxyribonucleotides* (p. 610). The conversion of L-methylmalonyl CoA into succinyl CoA (an intramolecular rearrangement) and the formation of methionine by methylation of homocysteine are the only known reactions dependent on coenzyme B_{12} in mammals.

The rearrangement reactions catalyzed by coenzyme B_{12} are exchanges of two groups attached to adjacent carbon atoms (Figure 21-17). A hydrogen atom migrates from one carbon atom to the next, and an X group (such as the —CO—S—CoA group of methylmalonyl CoA) concomitantly moves in the reverse direction. The first step in these intramolecular rearrangements is the cleavage of the carbon–cobalt bond of 5′-deoxyadenosylcobalamin to form B_{12r} (Co^{2+}) and a 5′-deoxyadenosyl radical (—$CH_2\cdot$) (Figure 21-18). In this *homolytic cleavage reaction*, one electron of the Co–C bond stays with Co and the other with C, generating a free radical. In contrast, nearly all other cleavage reactions in biological systems are *heterolytic*—an electron pair is transferred to one of the two atoms that were bonded together.

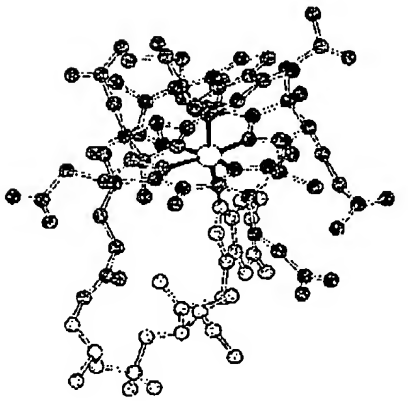


Figure 21-19
Model of coenzyme B_{12}. The cobalt atom is shown in yellow, the corrin unit in red, the 5′-deoxyadenosyl unit in blue, and the benzimidazole unit in green.

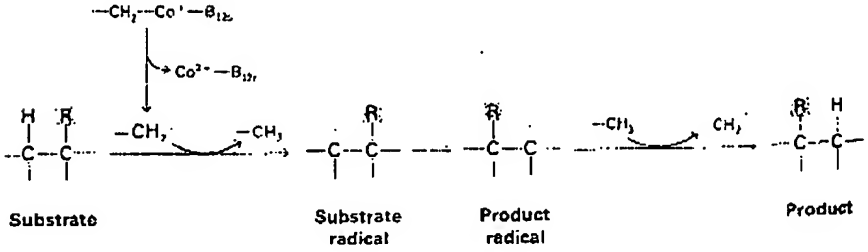


Figure 21-18
Coenzyme B_{12} provides the free radical that abstracts a hydrogen atom in rearrangement reactions.

Why is this very unusual —$CH_2\cdot$ radical formed? This highly reactive species abstracts a *hydrogen atom* from the substrate to form 5′-deoxyadenosine (—CH_3) and a substrate radical. This sets the stage for the migration of X to the position formerly occupied by H on the neighboring carbon atom. Finally, the product radical abstracts a hydrogen atom from the 5′-methyl group to complete the rearrangement

and return the deoxyadenosyl unit to the radical form. *The role of B_{12} in such intramolecular migrations is to serve as a source of free radicals for the abstraction of hydrogen atoms. A key property of coenzyme B_{12} is the weakness of its cobalt–carbon bond, whose facile cleavage generates a radical.* Steric crowding around the cobalt atom prevents the formation of a stronger bond, which would make the coenzyme a less effective catalyst.

ABSORPTION OF COBALAMIN IS IMPAIRED IN PERNICIOUS ANEMIA

Cobalamin is absorbed by a specialized transport system. The stomach secretes a glycoprotein called *intrinsic factor* (59 kd), which binds cobalamin in the intestinal lumen. This complex is subsequently bound by a *specific receptor in the lining of the ileum.* The complex of cobalamin and intrinsic factor is then dissociated by a *releasing factor* and actively transported across the ileal membrane into the bloodstream. *Pernicious anemia is caused by a deficiency of intrinsic factor, which leads to impaired absorption of cobalamin.* Consequently, the synthesis of purines and of thymine is impaired. This disease was originally treated by feeding patients large amounts of liver, a rich source of cobalamin, so that enough of the vitamin was absorbed even in the absence of intrinsic factor. The most reliable therapy is intramuscular injection of cobalamin at monthly intervals.

Animals and plants are unable to synthesize cobalamin. *This vitamin is unique in being synthesized only by microorganisms,* in particular, anaerobic bacteria. A normal person requires less than 10 μg of cobalamin per day. Nutritional deficiency of cobalamin is rare because this vitamin is found in virtually all animal tissues.

SEVERAL INHERITED DEFECTS OF METHYLMALONYL COENZYME A METABOLISM ARE KNOWN

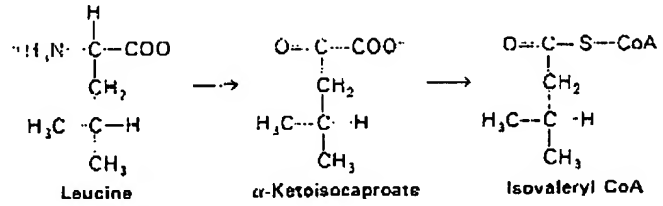
Several *inherited disorders of methylmalonyl CoA metabolism* have been characterized. They usually become evident in the first year of life, when the striking symptom is *acidosis.* The pH of arterial blood is about 7.0, rather than the normal value of 7.4. *Large amounts of methylmalonate appear in the urine of patients who have these disorders.* A normal person excretes less than 5 mg of methylmalonate per day, whereas a patient with defective methylmalonyl CoA metabolism may excrete more than 1 g. About half of the patients with methylmalonic aciduria improve markedly when large doses of cobalamin are administered intramuscularly. The arterial blood pH returns to normal, and there is a marked decrease in the excretion of methylmalonate. These responsive patients usually have a defect in the transferase that catalyzes the synthesis of coenzyme B_{12} from B_{12s} and ATP.

$$B_{12s}\ (Co^{+}) \dashv\vdash\longrightarrow \text{deoxyadenosylcobalamin}$$

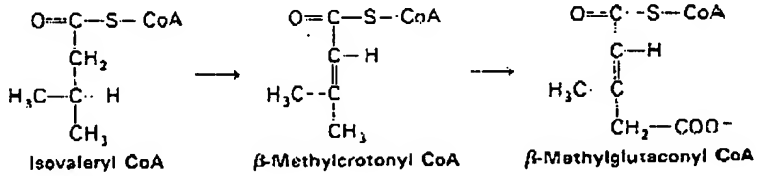
In contrast, other patients with impaired methylmalonyl CoA metabolism do not respond to large doses of cobalamin. Some of them may have a defective methylmalonyl CoA mutase apoenzyme. This form of methylmalonic aciduria is frequently lethal.

LEUCINE IS DEGRADED TO ACETYL COENZYME A AND ACETOACETYL COENZYME A

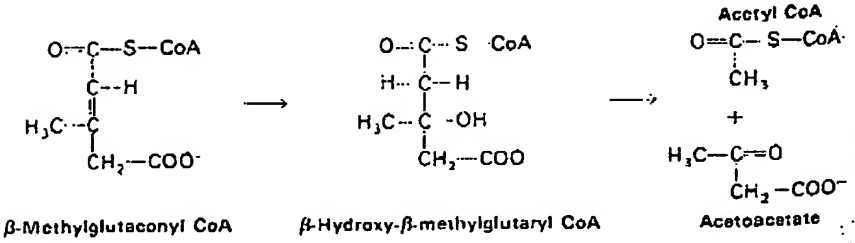
As was mentioned earlier, leucine and lysine are the only purely ketogenic amino acids of the common set of twenty. First, leucine is transaminated to the corresponding α-keto acid, *α-ketoisocaproate*. This α-keto acid is then degraded by reactions akin to those occurring in the citric acid cycle and fatty acid oxidation. It is *oxidatively decarboxylated* to *isovaleryl CoA*. This reaction is analogous to the oxidative decarboxylation of pyruvate to acetyl CoA and of α-ketoglutarate to succinyl CoA.



Isovaleryl CoA is *dehydrogenated* to yield *β-methylcrotonyl CoA*. This oxidation is catalyzed by isovaleryl CoA dehydrogenase, in which the hydrogen acceptor is FAD, as in the analogous reaction in fatty acid oxidation that is catalyzed by acyl CoA dehydrogenase. *β-Methylglutaconyl CoA* is formed by *carboxylation* of β-methylcrotonyl CoA at the expense of an ATP. As might be expected, the mechanism of carboxylation of β-methylcrotonyl CoA carboxylase is very similar to that of pyruvate carboxylase and acetyl CoA carboxylase. In fact, much of our present knowledge of the mechanism of biotin-dependent carboxylations comes from Feodor Lynen's pioneering work on this enzyme.



β-Methylglutaconyl CoA is then *hydrated* to form *β-hydroxy-β-methylglutaryl CoA*, which is cleaved to *acetyl CoA* and *acetoacetate*. This reaction has already been discussed in regard to the formation of ketone bodies from fatty acids (p. 479).



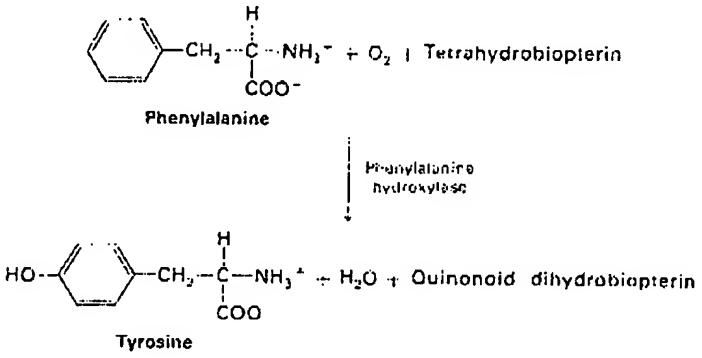
It is interesting to note that many coenzymes participate in the degradation of leucine to acetyl CoA and acetoacetate: *PLP* in transamina-

tion; *TPP, lipoate, FAD, and NAD*$^+$ in oxidative decarboxylation; FAD again in dehydrogenation; and *biotin* in carboxylation. *Coenzyme A* is the acyl carrier in these reactions.

The degradative pathways of valine and isoleucine resemble that of leucine. All three amino acids are initially transaminated to the corresponding α-keto acid, which is then oxidatively decarboxylated to yield a derivative of CoA. The subsequent reactions are like those of fatty acid oxidation. Isoleucine yields acetyl CoA and propionyl CoA, whereas valine yields methylmalonyl CoA. There is an inborn error of metabolism that affects the degradation of valine, isoleucine, and leucine. In *maple syrup urine disease*, the oxidative decarboxylation of these three amino acids is blocked. The amounts of leucine, isoleucine, and valine in blood and urine are markedly elevated, which results in a corresponding increase in the α-keto acids derived from these amino acids. The urine of patients having this disease has the odor of maple syrup, hence the name of the disease. Maple syrup urine disease is usually fatal unless the patient is placed on a diet low in valine, isoleucine, and leucine early in life.

PHENYLALANINE AND TYROSINE ARE DEGRADED BY OXYGENASES TO ACETOACETATE AND FUMARATE

The pathway for the degradation of phenylalanine and tyrosine has some very interesting features. This series of reactions shows how *molecular oxygen is used to break an aromatic ring*. The first step is the hydroxylation of phenylalanine to tyrosine, a reaction catalyzed by *phenylalanine hydroxylase*. This enzyme is called a *monooxygenase* (also called a *mixed-function oxygenase*) because *one atom of O_2 appears in the product and the other in H_2O.*



The reductant here is *tetrahydrobiopterin*, an electron carrier that has not been previously discussed. Tetrahydrobiopterin is initially formed by reduction of dihydrobiopterin by NADPH, in a reaction catalyzed by dihydrofolate reductase (Figure 21-20). The quinonoid form of dihydrobiopterin produced in the hydroxylation of phenylalanine is reduced back to tetrahydrobiopterin by NADH in a reaction catalyzed by dihydropteridine reductase. The sum of the reactions catalyzed by phenylalanine hydroxylase and dihydropteridine reductase is

$$\text{Phenylalanine} + O_2 + \text{NADH} + H^+ \longrightarrow \text{tyrosine} + \text{NAD}^+ + H_2O$$

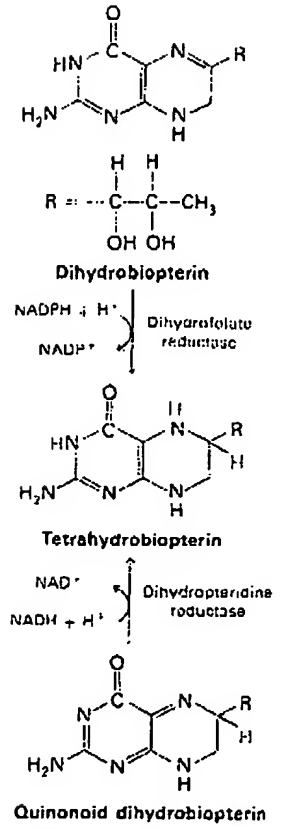


Figure 21-20
Formation of tetrahydrobiopterin by reduction of either of two forms of dihydrobiopterin.

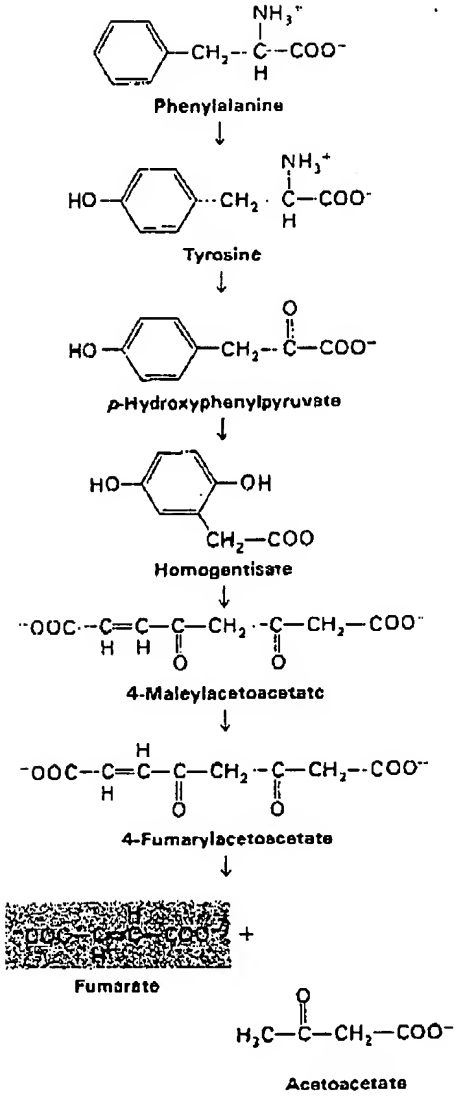


Figure 21-21
Pathway for the degradation of phenylalanine and tyrosine.

The next step is the transamination of tyrosine to p-*hydroxyphenylpyruvate* (Figure 21-21). This α-keto acid then reacts with O_2 to form *homogentisate*. The enzyme catalyzing this complex reaction, *p*-hydroxyphenylpyruvate hydroxylase, is called a *dioxygenase* because both atoms of O_2 become incorporated into the product, one on the ring and one in the carboxyl group. The aromatic ring of homogentisate is then cleaved by O_2, which yields 4-maleylacetoacetate. This reaction is catalyzed by homogentisate oxidase, another dioxygenase. 4-Maleylacetoacetate is then isomerized to *4-fumaryl acetoacetate* by an enzyme that uses glutathione as a cofactor. Finally, 4-fumarylacetoacetate is hydrolyzed to *fumarate* and *acetoacetate*.

We previously encountered a dioxygenase in the hydroxylation of proline (p. 263). Recall that α-ketoglutarate participates in this reaction and that one atom of O_2 emerges in hydroxyproline and the other in succinate. Prolyl hydroxylase and lysyl hydroxylase are *intermolecular dioxygenases* because they catalyze the incorporation of one atom of oxygen into each of *two* separate products. In contrast, *p*-hydroxyphenylpyruvate hydroxylase and homogentisate oxidase are *intramolecular dioxygenases* because both atoms of O_2 appear in the same product (Figure 21-22). The active sites of these enzymes contain iron that is not part of heme or an iron-sulfur cluster. *Nearly all cleavages of aromatic rings in biological systems are catalyzed by dioxygenases*, a class of enzymes discovered by Osamu Hayaishi.

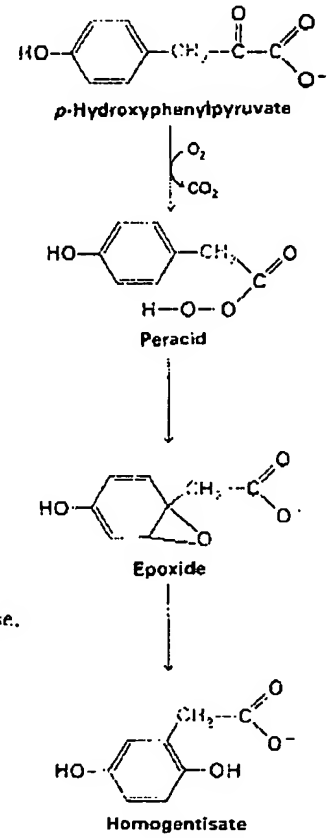


Figure 21-22
Formation of homogentisate. *p*-Hydroxyphenylpyruvate hydroxylase, the enzyme catalyzing this reaction, is an intramolecular dixoygenase. Both atoms of O_2 emerge in homogentisate.

GARROD'S DISCOVERY OF INBORN ERRORS OF METABOLISM

Alcaptonuria is an inherited metabolic disorder caused by the absence of homogentisate oxidase. Homogentisate accumulates and is excreted in the urine, which turns dark on standing as homogentisate is oxidized and polymerized to a melaninlike substance. Alcaptonuria is a relatively benign condition, as described by Zacutus Lusitanus in 1649:

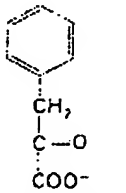
> The patient was a boy who passed black urine and who, at the age of fourteen years, was submitted to a drastic course of treatment which had for its aim the subduing of the fiery heat of his viscera, which was supposed to bring about the condition in question by charring and blackening his bile. Among the measures prescribed were bleedings, purgation, baths, a cold and watery diet and drugs galore. None of these had any obvious effect, and eventually the patient, who tired of the futile and superfluous therapy, resolved to let things take their natural course. None of the predicted evils ensued, he married, begat a large family, and lived a long and healthy life, always passing urine black as ink.

In 1902, Archibald Garrod showed that alcaptonuria is transmitted as a single recessive Mendelian trait. Furthermore, he recognized that homogentisate is a normal intermediate in the degradation of phenylalanine and tyrosine and that it accumulates in alcaptonuria because its degradation is blocked. He concluded that "the splitting of the benzene ring in normal metabolism is the work of a special enzyme, that in congenital alcaptonuria this enzyme is wanting." Garrod perceived the direct relationship between genes and enzymes, and he recognized the importance of chemical individuality. His book *Inborn Errors of Metabolism* was a most imaginative and important contribution to biology and medicine.

A BLOCK IN THE HYDROXYLATION OF PHENYLALANINE CAN LEAD TO SEVERE MENTAL RETARDATION

Phenylketonuria (PKU), an inborn error of phenylalanine metabolism, can have devastating effects, in contrast with alcaptonuria. Almost all untreated individuals with phenylketonuria are *severely mentally retarded.* In fact, about 1% of patients in mental institutions have phenylketonuria. The weight of the brain of these individuals is below normal, myelination of their nerves is defective, and their reflexes are hyperactive. The life expectancy of untreated phenylketonurics is drastically shortened. Half are dead by age twenty, and three-quarters by age thirty.

Phenylketonuria is caused by an *absence or deficiency of phenylalanine hydroxylase* or, more rarely, of its tetrahydrobiopterin cofactor. Phenylalanine cannot be converted into tyrosine, and so there is an accumulation of *phenylalanine in all body fluids.* Some minor fates of phenylalanine in normal persons become prominent in phenylketonurics. The most evident one is the transamination of phenylalanine to form *phenylpyruvate.* The disease acquired its name from the high levels of this phenylketone in urine. Phenyllactate, phenylacetate, and *o*-hydroxylphenylacetate are derived from phenylpyruvate. The α-amino group of glutamine forms an amide bond with the carboxyl group of phenylacetate, which yields phenylacetylglutamine.



Phenylpyruvate

There are many other derangements of amino acid metabolism associated with phenylketonuria, particularly among the aromatic compounds. Phenylketonu-

Melanin—
A black pigment in skin and hair. From the Greek word *melan*, meaning black. This polymeric pigment is formed in granules, called melanosomes, that are rich in *tyrosinase*, a monoxygenase.

rics have a lighter skin and hair color than their siblings. The hydroxylation of tyrosine is the first step in the formation of the pigment melanin. In phenylketonurics this reaction is competitively inhibited by the high levels of phenylalanine, and so less melanin is formed.

Phenylketonurics appear normal at birth but are severely defective by age one if untreated. The therapy for phenylketonuria is a *low phenylalanine diet*. The aim is to provide just enough phenylalanine to meet the needs for growth and replacement. Proteins that have an initially low content of phenylalanine, such as casein from milk, are hydrolyzed and phenylalanine is removed by adsorption. A low phenylalanine diet must be started very soon after birth to prevent irreversible brain damage. In one study, the average I.Q. of phenylketonurics treated within a few weeks after birth was 93; a control group of siblings treated starting at age one had an average I.Q. of 53. *However, the biochemical basis of mental retardation in untreated phenylketonuria is an enigma.*

Early diagnosis of phenylketonuria is essential and has been accomplished by mass screening programs. In past years, the urine of newborns was assayed by the addition of $FeCl_3$, which gives an olive green color in the presence of phenylpyruvate. The phenylalanine level in the blood is now the preferred diagnostic criterion because it is more reliable. Furthermore, the gene for human phenylalanine hydroxylase has recently been cloned, so that prenatal diagnosis of phenylketonuria is now feasible with DNA probes. The incidence of phenylketonuria is about 1 in 20,000 newborns. The disease is inherited as an *autosomal recessive*. Heterozygotes, which comprise about 1.5% of a typical population, appear normal. Carriers of the phenylketonuric gene have a reduced level of phenylalanine hydroxylase, as reflected in an increased level of phenylalanine in the blood. However, these criteria are not absolute, because the blood levels of phenylalanine in carriers and normal persons overlap to some extent. The measurement of the kinetics of disappearance of intravenously administered phenylalanine is a more definitive test for the carrier state. It should be noted that a high blood level of phenylalanine in a pregnant woman can result in abnormal development of the fetus. This is a striking example of maternal-fetal relationships at the molecular level.

SUMMARY

Surplus amino acids are used as metabolic fuel. The degradation of most surplus amino acids starts with the removal of their α-amino groups by transamination to an α-keto acid. Pyridoxal phosphate is the coenzyme in all aminotransferases and in many other enzymes catalyzing transformations of amino acids. The α-amino groups funnel into α-ketoglutarate to form glutamate, which is then oxidatively deaminated by glutamate dehydrogenase to give NH_4^+ and α-ketoglutarate. NAD^+ or $NADP^+$ is the electron acceptor in this reaction. In terrestrial vertebrates, NH_4^+ is converted into urea by the urea cycle. Urea is formed by the hydrolysis of arginine. The subsequent reactions of the urea cycle synthesize arginine from ornithine, the other product of the hydrolysis reaction. First, ornithine is carbamoylated to citrulline by carbamoyl phosphate. Citrulline then condenses with aspartate to form argininosuccinate, which is cleaved to arginine and fumarate. The car-

bon atom and one nitrogen atom of urea come from carbamoyl phosphate, which is synthesized from CO_2, NH_4^+, and ATP. The other nitrogen atom of urea comes from aspartate.

The carbon atoms of degraded amino acids are converted into pyruvate, acetyl CoA, acetoacetate, or an intermediate of the citric acid cycle. Most amino acids are purely glucogenic, two are purely ketogenic, and a few are both ketogenic and glucogenic. Alanine, serine, cysteine, glycine, and threonine are degraded to pyruvate. Asparagine and aspartate are converted into oxaloacetate. α-Ketoglutarate is the point of entry for glutamate and four amino acids (glutamine, histidine, proline, and arginine) that can be converted into glutamate. Succinyl CoA is the point of entry for some of the carbon atoms of three amino acids (methionine, isoleucine, and valine) that are degraded by way of methylmalonyl CoA. 5'-Deoxyadenosylcobalamin, a coenzyme formed from vitamin B_{12} and ATP, is the free-radical source in the isomerization of methylmalonyl CoA to succinyl CoA. Leucine is degraded to acetoacetyl CoA and acetyl CoA.

The aromatic rings of tyrosine and phenylalanine are degraded by oxygenases. Phenylalanine hydroxylase, a monooxygenase, uses tetrahydrobiopterin as the reductant. One of the oxygen atoms of O_2 emerges in tyrosine and the other in water. Absence or inactivity of this enzyme causes phenylketonuria; mental retardation results unless a low phenylalanine diet is started in infancy. Subsequent steps in the degradation of these aromatic amino acids are catalyzed by intramolecular dioxygenases that catalyze the insertion of both atoms of O_2 into a single product. Four of the carbon atoms of phenylalanine and tyrosine are converted into fumarate, and four emerge in acetoacetate.

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PROBLEMS

1. Name the α-keto acid that is formed by transamination of each of the following amino acids:
   (a) Alanine. (d) Leucine.
   (b) Aspartate. (e) Phenylalanine.
   (c) Glutamate. (f) Tyrosine.

2. Write a balanced equation for the conversion of aspartate into glucose by way of oxaloacetate. Cite the coenzymes that participate in these steps.

3. Write a balanced equation for the conversion of aspartate into oxaloacetate by way of fumarate.

4. Consider the mechanism of the conversion of L-methylmalonyl CoA into succinyl CoA by L-methylmalonyl CoA mutase.
   (a) Design an experiment to distinguish between the migration of the COO^- group and that of the —CO—S—CoA group in this reaction.
   (b) What is the significance of the finding that no tritium is incorporated into succinyl CoA when the mutase reaction is carried out in tritiated water?

5. Pyridoxal phosphate stabilizes carbanionic intermediates by serving as an electron sink. Which other prosthetic group catalyzes reactions in this way?

6. Propose a role for the positively charged guanido nitrogen in the cleavage of argininosuccinate into arginine and fumarate.

7. Methylmalonyl mutase is incubated with deuterated methylmalonyl CoA. Coenzyme B_{12} extracted from mutase in this reaction mixture is found to contain deuterium in its 5′-methylene group. Account for the transfer of label from substrate to coenzyme.

8. Heterolytic cleavage of a C–H bond can yield two types of products. What are they?